

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/11, A61K 31/70</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/06192</b> <b>(43) International Publication Date:</b> 16 April 1992 (16.04.92)
<b>(21) International Application Number:</b> PCT/US91/07550 <b>(22) International Filing Date:</b> 9 October 1991 (09.10.91) <b>(30) Priority data:</b> 595,253 9 October 1990 (09.10.90) US <b>(71) Applicant:</b> VESTAR, INC. [US/US]; 650 Cliffside Drive, San Dimas, CA 91773 (US). <b>(72) Inventors:</b> SCOLARO, Michael, J. ; 803 North Rexford Drive, Beverly Hills, CA 90210 (US). SULLIVAN, Sean, M. ; 2871 Sedgewick Road, Shaker Heights, OH 44120 (US). <b>(74) Agent:</b> COCHRAN, Adam; Vestar, Inc., 650 Cliffside Drive, San Dimas, CA 91773 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> INHIBITION OF VIRAL REPLICATION  <b>(57) Abstract</b>  The intracellular delivery of a DNA oligonucleotide having the same sequence as a DNA coding strand of viral origin which codes for the production of a viral regulatory protein inhibits replication, in a cell, of a virus having a genome which becomes integrated in the host DNA of the cell.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU <sup>+</sup>	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE*	Germany	MC	Monaco	US	United States of America
DK	Denmark				

+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

## INHIBITION OF VIRAL REPLICATION

### Field of the Invention

This invention relates to the fields of biochemistry and medicine, more particularly to the delivery to target cells of oligonucleotides complementary to viral genes for the purpose of inhibiting viral replication.

### Background of the Invention

A virus is an infectious agent composed of a protein sheath surrounding a nucleic acid core, which is capable of infecting a plant, animal or bacteria. Viruses are characterized by having a total dependence on a living host cell for reproduction, and by a lack of independent metabolism. After passing into the host cell, during an early stage of viral replication, the virion (*i.e.*, the complete, mature virus particle) disintegrates, freeing elements that direct the host cell's metabolism in the process of reproducing the virus. Specifically, the nucleic acid core (RNA or DNA) is released as the basic infectious material of the virus. Generally, virions containing DNA are referred to by the common term viruses, or more properly DNA viruses. Those containing RNA in the virion core, and whose genomic RNA is copied to form DNA which is integrated into the host DNA, are referred to as retroviruses or RNA viruses.

When DNA from a DNA virus is released in the host cell, this DNA is integrated into the cellular DNA for later reproduction of the virion which, often after destroying the host cell is expelled to continue the infection in other cells. If positive strand RNA is the genetic material of the virus, this RNA is copied to DNA by one of the viral proteins (reverse transcriptase) and the resulting viral DNA is integrated and reproduced by the reproductive mechanism of the host cell as described in the preceding sentence. For RNA viruses in which the genomic material does not integrate into the host DNA, both positive and negative stranded viral RNA are ultimately translated into structural proteins and copied into host cell genomic RNA by a viral RNA-dependent RNA polymerase for packaging into virions with no requirement for integration into cellular DNA.

A virus which is of much concern at present is the causative agent of autoimmune deficiency syndrome, which has been denominated as the human

immunodeficiency virus (HIV). This is an RNA virus whose genome is copied and becomes integrated into the host DNA. This virus has been shown to infect cells of the immune system, predominantly T cells, monocytes and neural tissue, resulting in a significant attenuation or destruction of immune response in the human body.

5 HIV first infects cells of the immune system by binding to the CD4 protein expressed on the membrane surface of the cell. The binding event initiates fusion of the viral membrane with the plasma membrane of the cell, releasing the viral core into the cytoplasm. The viral core contains a protein (reverse transcriptase) which copies the viral RNA into viral DNA. The viral DNA complexed with other viral  
10 proteins migrates to the cellular nucleus where the viral DNA is integrated into the genomic DNA of the host cell. This is termed the latent state of the infection because no virus particles are produced.

In the acute state of the infection, viral RNA is produced by transcription of the integrated viral DNA by a cellular enzyme termed RNA polymerase II. This  
15 RNA is processed into genomic RNA and messenger RNA. The messenger RNA is translated into structural proteins necessary for infectious virion assembly. The virions, composed of genomic RNA and the viral structural proteins, are assembled and released from the cell by budding. In addition, the HIV viral genome codes for six accessory genes which are not comprised within the virion but which are  
20 necessary for infectious virion assembly. These proteins are referred to as *tat* (*trans*-activating protein), *rev* (a regulator of virion protein expression), *nef*, *vif*, *vpr*, and *vpu*. The nucleic acid coding sequences for these proteins are encoded within the gene sequence coding for the envelope protein, which is a structural (as opposed to regulatory) virion protein. The envelope gene DNA is transcribed by RNA  
25 polymerase II to RNA, which is excised and ligated, leading to the production of mature messenger RNA which is transported to the cytoplasm and translated into the accessory proteins. The *tat* protein is a *trans*-activation protein which amplifies the production of viral RNA by binding to the promoter region of the integrated viral DNA. Furthermore, this protein also increases the translation of the mature  
30 messenger RNA. Loss of *tat* function results in completely non-infectious virus in which no detectable viral RNA or proteins can be expressed.

The open reading frame of the *rev* protein overlaps with that of the *tat* but the role of *rev* in the regulation of virus production is quite different. *Rev* prevents the transcribed viral RNA from being processed into accessory gene products, thus resulting in increased production of virion structural proteins necessary for assembly of infectious virion. Hence, loss of *rev* function results in the loss of infectious virion assembly capability.

One aspect of the development of agents to inhibit viral infections has focused on prophylactic treatment, *i.e.*, a vaccine or molecule which interacts solely with the viral machinery to inhibit virus particle production. The vaccines require the use of inactive virus particles as a source of antigen, which could lead to an allergic response to the contaminating proteins and the potential for incomplete inactivation. Further, viral mutations may require a continuing update of the antigen, as in the case of influenza vaccines.

Other workers have focused on the development of antiviral molecules which inhibit viral replication, particularly in DNA and RNA viruses which utilize their own polymerase for genome replication. The most successful drugs have been acyclovir and ganciclovir, which inhibit herpes simplex virus I and II and cytomegalovirus thymidine kinase. This strategy has been applied to HIV yielding a variety of nucleoside analogs with varying degrees of inhibition of replication. Of these, azidothymidine (AZT) has had success in reducing the production of virus in the bloodstream and increasing T-helper cell population. However, AZT is toxic to bone marrow and other cells and the treatment is dose limited. Further, the use of AZT is often incompatible with other antiinfectious agents (*e.g.* antifungal agents). Thus, treatment of opportunistic infections which result from immunosuppression require that AZT be discontinued until the infection is cleared.

Synthetic oligonucleotide therapy has been proposed for the treatment of such viral infections, for example, synthetic deoxyribonucleic acid sequences which are complementary to viral messenger ribonucleic acid (mRNA) have been shown to inhibit virus production in infected cells. This approach employs what has been termed antisense DNA because the synthetic DNA, usually an oligomer 20 to 40 sequences in length (20mer to 40mer), is complementary to (the opposite of) the coding strand of the proviral DNA or the viral mRNA.

The proposed mechanism of action is that the antisense DNA would bind to the viral mRNA resulting in a DNA-RNA duplex. This would result in the degradation (cleavage) of the RNA portion of the duplex by an intracellular enzyme called RNase H. As a result of this cleavage, it would no longer be possible for the mRNA to participate in protein synthesis. The synthetic antisense DNA would then be freed and available to bind to another mRNA strand.

These sequences are being applied to the treatment of disease. The drawbacks have been that oligonucleotides are susceptible to degradative enzymes existing in the circulation, intravenous administration results in rapid clearance from the bloodstream by the kidney, and uptake is insufficient to produce an effective intracellular drug concentration.

Liposomes are particles of subcellular size, comprised of one or more spherical lipid bilayers which surround an internal aqueous space. Drugs may be encapsulated either within the internal aqueous space or in the lipid bilayer, which is usually composed of a phospholipid such as saturated or unsaturated phosphatidylcholine, some type of sterol, and various other, charged or neutral, natural or synthetic lipids. The employment of a liposome delivery system can theoretically overcome the above mentioned problems in the administration of nucleic acid sequences. Liposome encapsulation protects the oligonucleotides from the degradative enzymes, increases the circulation half-life and increases uptake efficiency as a result of phagocytosis of the liposomes.

Phospholipids are amphipathic molecules which are the primary constituents of cell membranes. Typical phospholipid hydrophilic groups include phosphatidylcholine and phosphatidylethanolamine moieties, while typical hydrophobic groups include a variety of saturated and unsaturated fatty acid moieties. Mixture of a phospholipid in water causes spontaneous organization of the phospholipid molecules into a variety of characteristic phases depending on the conditions used. These include bilayer structures in which the hydrophilic groups of the phospholipids interact at the exterior of the bilayer with water, while the hydrophobic groups interact with similar groups on adjacent molecules in the interior of the bilayer. Such bilayer structures can be quite stable and form the principal basis for cell membranes.

Phospholipid bilayer structures can also be formed into closed spherical shell-like structures which are called phospholipid vesicles or liposomes. The membrane bilayers in these structures typically encapsulate an aqueous volume, and form a permeability barrier between the encapsulated volume and the exterior solution.

5 Phospholipids dispersed in aqueous solution spontaneously form bilayers with the hydrocarbon tails directed inward and the polar headgroups outward to interact with water. Simple agitation of the mixture usually produces multilamellar vesicles (MLVs), structures with many bilayers in an onion-like form having diameters of 1-10  $\mu\text{m}$  (1000-10,000 nm). Sonication of these structures, or other methods known

10 in the art, leads to formation of unilamellar vesicles (UVs) having an average diameter of about 30-300 nm. However, the range of 50 to 100 nm is considered to be optimal from the standpoint of, e.g., maximal circulation time *in vivo*. The actual equilibrium diameter is largely determined by the nature of the phospholipid used and the extent of incorporation of other lipids such as cholesterol. Standard

15 methods for the formation of liposomes are known in the art, for example, methods for the commercial production of liposomes are described in U.S. Patent No. 4,753,788 to Ronald C. Gamble and Patent No. 4,935,171 to Kevin R. Bracken, the disclosures of which are incorporated herein by reference.

Either as MLVs or UVs, liposomes have proven valuable as vehicles for drug

20 delivery in animals and in humans. Active drugs, including small hydrophilic molecules and polypeptides, can be trapped in the aqueous core of the liposome, while hydrophobic substances can be dissolved in the liposome membrane. The liposome structure can be readily injected and form the basis for both sustained release and drug delivery to specific cell types, or parts of the body. MLVs,

25 primarily because they are relatively large, are usually rapidly taken up by the reticuloendothelial system (the liver and spleen). UVs, however, tend to remain in the circulatory system for hours and break down after internalization by the target cell. Liposome mediated delivery of antisense DNA and DNA analogs have been used in attempts to inhibit viral proliferation.

Summary of the Invention

While the prior art has demonstrated the principle of using antisense DNA to inhibit virus production, the present invention comprises the delivery of sense DNA, preferably in liposome encapsulated form, to inhibit virus proliferation in infected cells and to serve as a prophylactic treatment to uninfected cells. Preferably, the sense oligonucleotide is encapsulated in UVs having a diameter of less than 200 nm, most preferably less than 100 nm.

Broadly, the method for the inhibition of viral replication in a cell comprises the intracellular delivery of a DNA oligonucleotide having the same sequence as a DNA coding strand of viral origin. Preferably, the sense oligonucleotide has the same sequence as the viral DNA coding strand for the production of a viral regulatory protein, particularly a *trans*-activating protein, and most preferably the DNA coding strand is a splice site necessary for the production of infectious virions. Significant advantages are obtained in a method in which the regulatory protein is *tat* or *rev*, in which the splice site includes the nucleotide sequence CAG, and in which the DNA oligonucleotide has the sequence 5'-ATTTTCAGAATTGGGTGTCG-3'. The method is applicable to viruses, has shown efficacy in retroviruses, particularly of the retroviridae family and the lentivirinae subfamily, and specifically useful in a method for inhibition of the replication of the human immunodeficiency virus. The invention provides significant advantages in the elimination of toxicity, since the DNA sequence is common only to the virus and has no effect on non-viral organisms, and the DNA sequence of the gene determines the specificity of the therapeutic. The method of the invention can inhibit HIV replication at the step of the conversion from viral RNA to DNA upon initial entry followed by inhibition of integration due to incomplete reverse transcription, or by the prevention of transcription of viral DNA to viral RNA. The sense oligonucleotide can be administered to infected cells *in vitro* or *in vivo* in amounts which are effective to treat the infection.

In addition to liposomes, other phospholipid delivery vehicles may be used to deliver sense oligos, such as those disclosed in the Vestar, Inc. patent publication EP0272091. This patent application, the counterpart of U.S. Serial No. 942,093 filed 15 December 1986, describes the use of a lipid particle which is composed of a

single encapsulating phospholipid membrane associated with an amphiphile-associated substrate.

#### Detailed Description of the Preferred Embodiment

The invention includes a method for use of sense DNA in the inhibition of viral proliferation in viruses that replicate through the integration of viral DNA, directly from the viral genome or transcribed from viral genomic RNA, into the host cell genome. In particular, the DNA employed is sense to the viral DNA coding strand which codes for the production of a viral regulatory protein.

Such viruses include viruses having accessory protein splice sites in the viral genome, adenovirus, herpes simplex viruses, Epstein Barr viruses, hepatitis virus, and papilloma virus. Examples of such accessory proteins are Ela protein for adenovirus, ICP4 protein for herpes simplex viruses, SM1, Z1 and R1 proteins for Epstein Barr viruses, and protein X for hepatitis virus. Other such viruses include the avian leukemia virus, the murine leukemia virus, the human T-cell leukemia virus, the human cytomegalovirus, and the human immunodeficiency viruses; all of which also include, in the viral DNA, a splice site necessary for the production of infectious virions.

The delivery of sense DNA, *i.e.*, an oligonucleotide having the same rather than a complementary sequence to the coding strand, offers several advantages over conventional antiviral treatments in that a sequence can be selected which is specific only to the virus and is therefore non-toxic to the cells. In addition, the selected target sequence can be one which is highly conserved in the viral genome to avoid viral resistance to the treatment as a result of natural viral mutation. While we do not wish to be bound to any particular theory, the synthetic sense DNA appears to be able to inhibit virus production at two stages in the viral cycle. The first consists of the binding of sense DNA to viral RNA directly after the uncoating of the virion. The second is the binding of the sense to the proviral DNA, thus preventing transcription. For the reasons mention above, the use of sense DNA which codes to the sequence for the production of a viral regulatory protein, a *trans*-activating protein, or a splice site necessary for the production of infectious virions is particularly advantageous in double-stranded DNA and RNA viruses.

Sense DNA which inhibits production of the *tat* protein is advantageous in cells infected with HIV because the elimination of this protein completely inhibits viral replication. Furthermore, *tat* has been shown to enter non-infected cells and inhibit the stimulation of immune cells by itself. Hence, by inhibiting *tat* protein  
5 production not only is the production of infectious virions inhibited, but also the secondary inhibition of the immune system by *tat* itself is inhibited.

In the examples which follow, a DNA sequence from the 5' splice acceptor site of the HIV-1 (human immunodeficiency virus) *trans*-activating protein termed *tat* was selected. The phosphodiester antisense sequence has been shown to inhibit  
10 HIV proliferation as determined by assaying for reverse transcriptase activity which is a characteristic enzyme activity of the virus. Inhibition of the HIV *tat* protein is highly desirable because upon production, this protein binds to the 5' long terminal repeat sequence of the integrated proviral DNA resulting in approximately 100 fold increase in viral protein production. Furthermore, since it is a regulatory gene, the  
15 copies of viral message should be much lower than copies of mRNA for the HIV structural genes (proteins which make up the virion).

The sense and the antisense sequences were encapsulated in small unilamellar liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL) and N-(N-ethylsuccinimidylthio)distearoylphosphatidylethanolamine (NEST-DSPE)  
20 in a ratio of 48/25/27 mol/mol. Specifically, a phosphatidylethanolamine (distearoylphosphatidylethanolamine (DSPE)) is attached to an acetylthio group by the addition of succinimidylacetylthioacetate (SATA) to form a phosphatidylethanolaminoacetylthioacetate (PE-ATA) moiety, specifically distearoylphosphatidylethanolaminoacetylthioacetate (DSPE-ATA). Liposomes are  
25 then formed (in ways which are within the knowledge of those of ordinary skill in the art) consisting of DPPC/Chol/DSPE-ATA, that is, liposomes consisting of DPPC/Chol/DSPE with an -ATA group extending externally from the surface thereof. After liposome formation, the -ATA moiety is deacetylated by addition of either hydroxylamine or ammonium hydroxide, and the free sulfhydryl of the -ATA  
30 is then reacted with N-ethylmaleimide to form the succinimidyl moiety on the DSPE, e.g., the NEST-DSPE. The succinimidyl moiety, extending from the surface of the liposome as the only significant extending group (in the absence of extending ligand

conjugates such as hormones or other proteins or peptides which extend from the lipid particle, *e.g.*, for the purpose of inducing a metabolic response in a target cell upon the binding of the ligand conjugate to its receptor), provides significant advantages in that it enables the cytoplasmic or nuclear cell delivery of intact  
5 oligonucleotides, to avoid degradation of the agents by the cell lysosome. Further information regarding this liposome formulation is found in the copending application entitled *Phospholipid Analogue Vesicle* which is filed on even date herewith, the disclosure of which is incorporated herein by reference.

Only the liposomes containing the sense sequence to the *tat* splice acceptor  
10 site inhibited virus production. The degree of inhibition has ranged from 50% to 85%. Furthermore, a 50% reduction in the number of infected cells was observed. Hence, the liposomes containing the sense DNA to the 5' splice acceptor site of the HIV *tat* gene were able to reduce viral production and inhibit the spread of the virus to uninfected cells.

15

## EXAMPLES

### Synthesis of DSPE-ATA

DSPE-ATA is synthesized by a reaction which generates a modified phosphatidylethanolamine having an acetylthioester group on the ethanolamine moiety. For example, a five-fold molar excess of SATA is added to DSPE in a  
20 round bottom flask. Typically, 125 mg SATA is added to 75 mg of DSPE. 15 ml of  $\text{CHCl}_3$ :MeOH (1:1) is then added, followed by 100-135  $\mu\text{l}$  of triethylamine. The flask is then flushed with nitrogen, sealed, and the reaction carried on for two hours at room temperature with stirring. The progress of the reaction is monitored by thin layer chromatography in  $\text{CHCl}_3$ :MeOH (7:3). The absence of a Ninhydrin positive,  
25 and the presence of a PMA positive spot having an  $R_f$  of 0.57 indicates the presence of DSPE-ATA. Some of this compound may oxidize to the disulfide (ATA-PE-ATA-PE), which has an  $R_f$  of about 0.37. It should be noted that SATA is quite labile and cannot be stored for prolonged periods. The material obtained is then evaporated to dryness and resuspended in 1.0 ml of  $\text{CHCl}_3$ :MeOH (1:1), and 15 ml  
30 of acetonitrile is added to the dry material. This solution is held at  $-20^\circ\text{C}$  for about 60 minutes to precipitate DSPE-ATA. The precipitate is collected by filtration on

a sintered glass filter and washed with acetonitrile. The washed DSPE-ATA is again dissolved by the addition of 1:1  $\text{CHCl}_3$ :MeOH and collected in a second filter flask, transferred to a preweighed flask and evaporated. The yield at this point has been between 80 and 84%.

5 During preparation, all steps should be conducted as quickly as possible to minimize oxidation. However, the removal of oxidized DSPE-ATA is accomplished by preparative thin layer chromatography, such as on a Kieselgel 60 plate obtained from EM Science. Aluminum backed silica gel (no fluorescent indicator) plates are used with  $\text{CHCl}_3$ :MeOH (7:3) as the mobile phase. DSPE-ATA is located by  $\text{I}_2$   
10 staining a strip cut from the end of the plate. The area containing DSPE-ATA is then cut out. The strips of  $\text{SiO}_2$ -coated aluminum are further cut up and extracted over a 30 minute period in 1:1  $\text{CHCl}_3$ :MeOH. The DSPE-ATA is concentrated to dryness, dissolved in 1:1  $\text{CHCl}_3$ :MeOH and dispensed into vials. Each vial is flushed with nitrogen and stored at  $-20^\circ\text{C}$ .

### 15 Liposome Production

A solution containing 15  $\mu\text{mol}$  of the lipids DPPC, DSPE-ATA and CHOL (49/27/25) was prepared in chloroform. The chloroform was evaporated under a stream of nitrogen to yield a dry powder or lipid film. The film was vacuum desiccated overnight to remove residual organic solvent. One film was hydrated in  
20 0.4 ml of phosphate buffered saline (PBS). Second and third films were hydrated in PBS containing either 2.5 mg of synthetic sense DNA (5'-ATTTTCAGAATTGGGTGTCG-3') or 2.5 mg of synthetic antisense DNA (5'-CGACACCCAATTCTGAAAAT-3'). For preparation of liposomes by extrusion, samples were brought through three freeze and thaw cycles using liquid nitrogen and  
25 thawing at  $65^\circ\text{C}$ . The suspensions were extruded through a  $0.4\mu\text{m}$ ,  $0.2\mu\text{m}$  and  $0.1\mu\text{m}$  polycarbonate filters three times per filter at  $65^\circ\text{C}$  and at a maximum of 800 psi to form liposomes having an average diameter of 100 nm. Each sample was extruded through a set of filters separately to avoid contamination and the order of extrusion was PBS, antisense DNA and sense DNA. Argon was bubbled through the  
30 lipid suspensions to remove oxygen. The thioacetylated lipid was deacetylated by addition of hydroxylamine. The byproducts were separated by a sephadex G-25 spin

column and the samples were incubated for 2.5 hours at room temperature. To attach a succinimide moiety to the liposome surface, N-ethylmaleimide was added to the liposome dispersion in a mol/mol excess of ten to one with respect to the thiolated lipid and incubated overnight at 4°C to yield the N-ethylsuccinimidylthiol phospholipid analogue. The byproducts were removed by a biogel A5M gel filtration column. The lipid fractions were pooled and sterile filtered using a 0.2 polycarbonate nucleopore filter. The preparation yielded liposomes of less than 100 nm in diameter, which contained approximately 140 molecules of each DNA per liposome.

#### 10 Tests of Viral Inhibition

The sterile liposome preparations produced as described in the preceding paragraph were added to human peripheral blood mononuclear cells (PBMCs) which had been cultured for three days in phytohemagglutinin (PHA). PBMCs from HIV positive infected patients were cultured with conditioned media to produce virus in the supernatant. This supernatant was collected and used to infect the PHA stimulated uninfected PBMCs. The liposomes were added 3 hours after the PBMCs were treated with the infected supernatant. Virus production was monitored by assaying the cell supernatant for HIV core protein termed *gag* protein or abbreviated p24. Levels of p24 were determined by a standard ELISA assay. The number of infected cells were determined by immunochemically treating fixed infected cultures with antibody to the HIV p17 and gp41 proteins (which also react with gp160 protein) followed by a secondary antibody to which an enzyme was linked. The infected cells were visualized by then incubating with a colored substrate to the enzyme followed by staining the uninfected cells.

25 The liposomes containing the 5'-ATTTTCAGAATTGGGTGTCG-3' DNA (having the same sequence as the DNA coding strand of viral origin, *i.e.*, the sense DNA) reduced the production of p24 into the cell supernatant by 85% with respect to the untreated control whereas liposomes containing the 5'-CGACACCCAATTCTGAAAAT-3' (having the opposite sequence of the DNA coding strand of viral origin, *i.e.*, the antisense DNA) showed no inhibition of p24 production. Immunocytochemical staining of the infected cells showed that the

liposomes containing the sense sequence reduced the number of strongly positive infected cells by 50% whereas the liposomes containing the antisense sequence showed no reduction in the number of strongly positive cells. It was also observed that the untreated control and liposomes containing the antisense sequence displayed large cell aggregates whereas the liposomes containing the sense sequence showed a greatly reduced number of large cell aggregates (approximately 30% of the untreated control). Neither the liposomal sense or liposomal antisense DNA sequence displayed cell toxicity as assayed by cell viability.

In a second set of experiments, free DNA was compared to liposomal DNA described above using two doses and testing for breakthrough of the virus on day 5 and day 7. Samples containing 50 and 100  $\mu$ M lipid were tested, which was equivalent to a concentration of 17 and 34 nM DNA. Liposomes containing the sense sequence showed 48% inhibition of p24 production at 50  $\mu$ M lipid and 50% inhibition at 100  $\mu$ M lipid on day 5 of infection. On day 7 the 50  $\mu$ M lipid showed 28% p24 inhibition and 31% inhibition at 100  $\mu$ M lipid. Liposomes containing the antisense sequence showed only 1.2% inhibition with 50 mM lipid and -9.6% inhibition with 100  $\mu$ M lipid on day 5. Day 7 showed 8% inhibition with 50  $\mu$ M and 5% inhibition with 100  $\mu$ M lipid.

Free 5'-ATTTTCAGAATTGGGTGTCG-3' (sense) yielded -8% inhibition with 17 nM DNA and 2% inhibition with 34 nM. 17% inhibition was observed for 17 nM and 13% inhibition was observed for 34 nM DNA on day 7. Empty liposomes showed no inhibition of p24 production. Furthermore, immunocytochemical staining for infected cells showed a decrease in the number of strongly positive infected cells and the number of giant cells by treatment with liposomes containing the sense DNA sequence compared to that observed for free sense, empty liposomes or liposomes containing the antisense sequence.

Finally, in a third set of experiments, DNA analogs of the above sequences were prepared containing modified 3' and 5' ends to increase resistance to nuclease degradation. These liposomes were prepared by first codissolving the DNA and lipid in chloroform/methanol (1:1) at a ratio of 0.5 mg DNA to 10  $\mu$ mol lipid. The solvent was evaporated forming a film on the side of the test tube, and the film was vacuum desiccated overnight to remove residual solvent. The films were hydrated

in 150 mM NaCl, 10 mM phosphate buffer. The hydrated films were then brought through three freeze and thaw cycles followed by the standard extrusion protocol previously described. Analysis showed a 23% trapping efficiency.

Empty liposomes formed by this method were tested against similar liposomes  
5 containing the above-described sense and antisense sequences. Liposomes were tested at 2, 10 and 50  $\mu$ M lipid and analyzed for inhibition of p24 production on day 5 and day 7. The following results were obtained for the sense liposomes containing the low, medium and high dose: on day 5: -8%, 11% and 42% of untreated control, respectively and on day 7: -6%, 28% and 60% of the untreated control. For the  
10 antisense liposomes, the results obtained for the low, medium and high dose on day 5 were 4%, 1% and 8% of the untreated control, respectively, and for day 7 the results were 2%, 11%, and 16% of the untreated control. Blocking the 3' and 5' ends of the oligonucleotides prevented oligonucleotide degradation and virus breakthrough on day 7, and shows the sequence specificity of the the splice site sense  
15 strand for the viral inhibition.

Nonspecific inhibition was observed for the empty liposomes as well, yielding 1%, 11% and 15% of the untreated control on day 5 and -60%, 18% and 28% of the untreated control on day 7 for the low, medium and high lipid doses.

Thus, liposomal sense DNA significantly reduces viral antigen production, p24  
20 levels, and the number of infected cells. DNA-free liposomes displayed some non-specific inhibition, which appears to account for the apparent inhibition seen by the liposomal antisense formulation, but the inhibition was significantly less than that shown by the sense liposomes.

## WE CLAIM:

1. A method for the inhibition of the replication, in a cell, of a virus having a genome which becomes integrated in the host DNA of the cell, comprising the intracellular delivery of a DNA oligonucleotide having the same sequence as a DNA  
5 coding strand of viral origin which codes for the production of a viral regulatory protein.
2. The method of claim 1 in which the DNA coding strand is a splice site necessary for the production of infectious virions.
3. The method of claim 1 or 2 in which the viral regulatory protein for which  
10 the DNA coding strand codes is a *trans*-activating element of viral origin.
4. The method of claim 3 in which the regulatory protein is *tat* or *rev*.
5. The method of claim 1 or 2 in which the splice site includes the nucleotide sequence CAG.
6. The method of claim 3 in which the splice site includes the nucleotide  
15 sequence CAG.
7. The method of claim 1 in which the DNA oligonucleotide has the sequence 5'-ATTTTCAGAATTGGGTGTCG-3'.
8. The method of claim 1, 2 or 7 in which the DNA oligonucleotide is encapsulated in liposomes.
- 20 9. The method of claim 3 in which the DNA oligonucleotide is encapsulated in liposomes.

10. The method of claim 4 in which the DNA oligonucleotide is encapsulated in liposomes.

11. The method of claim 5 in which the DNA oligonucleotide is encapsulated in liposomes.

5 12. The method of claim 6 in which the DNA oligonucleotide is encapsulated in liposomes.

13. The use, in the preparation of a composition for the inhibition of the intracellular replication of a virus having a genome which becomes integrated in the host DNA of the cell, of a DNA oligonucleotide having the same sequence as a DNA  
10 coding strand of viral origin which codes for the production of a viral regulatory protein.

14. The use of claim 13 in which the DNA coding strand is a splice site necessary for the production of infectious virions.

15. The use of claim 13 or 14 in which the viral regulatory protein for which  
15 the DNA coding strand codes is a *trans*-activating element of viral origin.

16. The use of claim 15 in which the regulatory protein is *tat* or *rev*.

17. The use of any one of claims 13 to 15 in which the splice site includes the nucleotide sequence CAG.

18. The use of claim 13 in which the DNA oligonucleotide has the sequence  
20 5'-ATTTTCAGAATTGGGTGTCG-3'.

19. The use of any one of claims 13 to 18 in which the DNA oligonucleotide is encapsulated in liposomes.

20. Use of liposomes for containing a DNA oligonucleotide, having the same sequence as a DNA coding strand of viral origin which codes for the production of a viral regulatory protein, to be delivered into a cell for the inhibition of the intracellular replication of a virus having a genome which becomes integrated in the host DNA of the cell.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/07550

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5                      C 12 N    I5/11                      A 61 K    31/70		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl.5	C 12 N                      A 61 K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>o</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Journal of Virology, volume 62, no. 10, October 1988, American Society for Microbiology (Baltimore, US) J.A. Zaia et al.: "Inhibition of human immunodeficiency virus by using an oligonucleoside methylphosphonate targeted to the tat-3 gene", pages 3914-3917, see the abstract; page 3916, right-hand column <div style="text-align: center;">---</div>	1-6, 13-17
Y	<div style="text-align: center;">---</div>	7-12, 18-20
X	DE, A, 3742049 (K.E. THEURER) 22 June 1989, see the whole document	20
Y	<div style="text-align: center;">---</div> <div style="text-align: center;">-/-</div>	8-12, 19-20
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>o</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
31-01-1992		24 FEB 1992
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		Mme N. KUIPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8908146 (WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 8 September 1989, see the whole document, especially page 35, table I and page 37, lines 1-18 ---	7,18
A	WO,A,8908146 ---	1-20
A	WO,A,8810311 (SYNTRO CORPORATION) 29 December 1988, see pages 37-44; claims ---	1-20
A	Biochemical and Biophysical Research Communications, volume 169, no. 2, 15 June 1990, Academic Press. Inc. (Duluth, Minnesota, US) G. Sczakiel et al.: "Specific inhibition of human immunodeficiency virus type 1 replication by RNA transcribed in sense and antisense orientation from the 5'-leader/gag region", pages 643-651, see the whole article ---	
A	Proceedings of the National Academy of Sciences of USA, volume 86, no. 20, October 1989 (Washington, DC, US) S. Agrawal et al.: "Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues", pages 7790-7794, see page 7791, table 1; oligonucleotides 1 and 3 -----	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:  
 "Remark: Although claims 1-12, 20 (as far as they concern in vivo delivery) are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:
3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9107550  
SA 53109

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/02/92. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A- 3742049	22-06-89	DE-A- 3744785	17-08-89
WO-A- 8908146	08-09-89	AU-A- 4072089	22-09-89
		EP-A- 0402402	19-12-90
WO-A- 8810311	29-12-88	AU-A- 2072388	19-01-89
		EP-A- 0319570	14-06-89